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Combined effect of local isolate *Spodoptera littoralis* nucleopolyhedrosis virus and *Bacillus thuringiensis* on *Culex pipiens* L. larvae (Culicidae: Diptera)

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Abstract A local isolate of *Spodoptera littoralis* nucleopolyhedrosis virus (*Sl*NPV) showed pathogenicity against *Culex pipiens* larvae, its long incubation period was broken using a combination between *Sl*NPV and *Bacillus thuringiensis subsp. kurstaki* (*Btk*). Laboratory bioassay tests revealed that *Culex pipiens* 3rd larval instars were susceptible to the applied combination. Data revealed that the addition of *Btk* generally increased the toxic effect of *Sl*NPV since LC_{50} decreased from 1.3×10^3 PIB/ml (for *Sl*NPV alone) to 3.6×10^2 mixed polyhedra and spores/ml (*Sl*NPV-*Btk* combination), indicating a synergistic ratio of 3.6. LC_{95} was surprisingly 137-folds dropped as well. The addition of *Sl*NPV generally increased the toxic effect of *Btk* at a low concentration, where the LC_{50} outstandingly decreased from 2.5×10^5 spores/ml (for *Btk*) to 3.6×10^2 mixed polyhedra and spores/ml (for *Sl*NPV-*Btk* combination), indicating a synergistic ratio of 6.9×10^2.

Introduction

Due to rapid increase in mosquito resistance and growing public concern over environmental pollution, use of chemical insecticides for mosquito control is no longer encouraged; rather use of effective and eco-friendly alternatives is promoted (Vincent, 2000; Hayes et al., 2011). In addition to microbial control agents' efficacy, they are safe for human and other non-target organisms, reduce the pesticide residues in food and preserve most of other natural enemies (Lacey et al., 2001).

Baculoviridae are a large family of entomopathogenic viruses whose double-stranded circular DNA infects many invertebrates, particularly Lepidoptera species (Moscardi, 1999). Virions are protected by the polyhedral occlusion bodies as they can retain infectivity for several years (Groner, 1987). Viral replication begins in the nuclei of mid-gut epithelial cells

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and infection spreads to various tissues until death of the insect (Thiem, 1997; Fuxa, 2004). A short time ago, there have been tremendous advancements in the ability to transmit mosquito Baculovirus with the finding that transmission is mediated by divalent cations. Oral transmission of the virus to mosquito larva is enhanced by magnesium (Becnel, 2006). The major restrictive factor for the application of NPV is relatively long-time between the infection and the death of the insect (Van Beek and Hughes, 1998). However, Baculoviruses can be combined with other entomopathogens to improve biological control of insect pests (Hesketh and Hails, 2008). Masetti et al. (2008) examined the enhancement of efficacy of microbial products for *Spodoptera littoralis* (*S. littoralis*) management by spraying combinations of *Bacillus thuringiensis* (*Bt*) toxins and *S. littoralis* NPV (*Sl*NPV).

Bacillus is a gram-positive bacterium that possesses a parasporal crystalline protein that is highly toxic to a wide-range of pest insects especially lepidopteran, dipteran, and coleopteran insects (Vettori et al., 2003; Mansour et al., 2012). *B. thuringiensis* produces parasporal crystals (inclusions) during its sporulation. These inclusions (δ-endotoxin) bind to specific receptors in the mid-gut brush border membrane of susceptible insects (Balaraman, 2005). *B. thuringiensis subsp. kurstaki* (*Btk*) is highly toxic to Lepidopteran larvae (Knowles et al., 1984; Perez-Guerrers et al., 2011). However, a previous study (Yamamoto and McLaughlin, 1981) reported that a certain strain of *Btk* has 2 distinct moieties, a lepidopteran specific toxin and a "mosquito factor" toxic to both mosquitoes and lepidopteran larvae.

A local strain of Baculovirus from laboratory infected *S. littoralis* larvae has been isolated. We tried to test the joint effect of such virus (*Sl*NPV) with *Btk* on *Cx. pipiens* larvae to achieve the following objectives: (1). Evaluate the potency of *Btk* and *Sl*NPV alone or in combination against mosquito larvae. (2). Reduce the relatively long incubation period of NPV which is an obstacle during application (Van Beek and Hughes, 1998). (3). Lowering the industrial costs by producing one product of *Bt* which can be used against lepidopteran and dipteran larvae at the same time.

Materials and methods

Insect rearing

Cx. pipiens larvae were obtained from Research Training Center and were identified according to Harbach (1988). Adult mosquitoes were reared in breeding cages under laboratory conditions of temperature (26 ± 2 °C), relative humidity (70–80% R.H), and 12 h photoperiod. Female mosquitoes were fed on blood of pigeon for egg deposition. Rearing was conducted according to the method described by Gad et al. (1988).

Nuclear polyhedrosis virus (NPV)

A laboratory NPV suspension was isolated from a laboratory strain 3rd larval instar of *S. littoralis*; obtained from Plant Protection Research Institute. These larvae were reared on castor bean leaf and collected after the appearance of symptoms of viral infection. The infected larvae were ground and the homogenate was suspended in 100 ml of 50 mM Tris/HCl buffer (pH 8), then filtrated through 4 layers of muslin. The filtrate was centrifuged at 1000 rpm for 4 min to precipitate larval debris, the supernatant was centrifuged at 12,000 rpm for 20 min and the pellet was resuspended in 50 mM Tris/HCl. For more purification, the virus suspension was centrifuged through linear sucrose gradient of 40–65% (w/w) at 14,000*g* for one hour at 20 °C, the virus bands were collected and washed in three volumes of distilled water, then centrifuged twice at 12,000 rpm for 20 min at 4 °C. The pellet was washed once again in Tris/HCl. The concentration of the virus was calculated by using hemocytometer then stored at −20 °C (Backwad and Pawar, 1981).

B. thuringiensis kurstaki (Btk)

Dipel, a wettable powder formulation of *Btk* was obtained from Plant Protection Research Institute, and was used to prepare serial dilutions for bioassay. Bacteria were assayed against 3rd instar larvae of *Cx. pipiens*.

Bioassay

Bioassays were accomplished in the same laboratory conditions mentioned above. Virus assays were applied to mosquito larvae using five different concentrations ranging from 10^5 to 10 polyhedra/ml. Ten mM ($MgCl_2$) was added for each treatment (Becnel, 2006). For bacterial bioassay tests, the concentrations ranged from 5×10^5 to 10^4 spores/ml. To the *Sl*NPV dilutions (10^5–10 polyhedra/ml), LC_{50} value of bacterial suspension was added. Ten mM ($MgCl_2$) was added for each treatment. Three replicates each of 20 larvae were assayed against each concentration. A Control group received distilled water free from viruses or bacteria. Mortality readings were recorded 24 h post-treatment. Rearing and bioassays were performed in Entomology Department, Faculty of Science, Ain Shams University.

Statistical analysis

Mortality readings were corrected according to Abbott's formula (1925), and lethal concentrations (LC_{50}, and LC_{95}) were calculated. Statistical analysis was carried out using the Probit Analysis Program (Finney, 1971). Synergistic ratios were calculated according to Fahmy and Miyata (1998).

Results

Tables 1 and 2 represent the LC values for laboratory isolates of *Sl*NPV, *Btk*, and *Sl*NPV-*Btk* combination. It is clear that the 3rd larval instars of *Cx. pipiens* were susceptible to the applied concentrations. Based on LC_{50}, *Sl*NPV-*Btk* combination was more active ($LC_{50} = 3.6 \times 10^2$ mixed polyhedra and spores/ml) than either *Sl*NPV ($LC_{50} = 1.3 \times 10^3$ PIB/ml) (Table 1) or *Btk* ($LC_{50} = 2.5 \times 10^5$ spores/ml) (Table 2) alone.

From Table 1, it appears that the addition of *Btk* generally increased the toxic effect of *Sl*NPV since LC_{50} decreased from 1.3×10^3 PIB/ml (for *Sl*NPV alone) to 3.6×10^2 mixed polyhedra and spores/ml (*Sl*NPV-*Btk* combination), indicating a synergistic ratio of 3.6. The LC_{95} was surprisingly 137-folds

Table 1 Susceptibility of *Cx. pipiens* third instar larvae to the nucleopolyhedrovirus of *S. littoralis* (*Sl*NPV) (laboratory isolate) and to combination of *Sl*NPV and *B. thuringiensis kurstaki* (*Btk*) 24 h post-infection with synergistic ratios.

LC values in PIB[a]*/ml (95% C.I.)*[b]		
Treatment	LC_{50} (95% C.I.)	LC_{95} (95% C.I.)
*Sl*NPV alone	1.3×10^3 (1.2×10^3–1.5×10^5)	2.2×10^9 (2.4×10^5–1×10^{14})
LC values in mixed PIB[a]*/ml and spores/ml (95% C.I.)*[b]		
*Sl*NP-*Btk* combination	3.6×10^2 (7.6×10–3.3×10^3)	1.6×10^7 (1.2×10^4–1.2×10^{11})
Synergistic ratio[c]	3.6	1.3×10^2

[a] PIB: polyhedral inclusion body.
[b] Ninety-five percent confidence limit.
[c] LC of *Sl*NPV/LC of (*Sl*NPV-*Btk*).

Table 2 Susceptibility of *Cx. pipiens* third instar larvae to *B. thuringiensis kurstaki* (*Btk*) and to combination of nucleopolyhedrovirus of *S. littoralis* (*Sl*NPV) (laboratory isolate) and *Btk*, 24 h post infection.

LC values in spores/ml (95% C.I.)[b]		
Treatment	LC_{50} (95% C.I.)	LC_{95} (95% C.I.)
Btk alone	2.5×10^5 (1×10^5–6.6×10^5)	1.3×10^7 (6.1×10^5–3×10^8)
LC values in mixed PIB[a]*/ml and spores/ml (95% C.I.)*[b]		
*Sl*NP-*Btk* combination	3.6×10^2 (7.6×10–3.3×10^3)	1.6×10^7 (1.2×10^4–1.2×10^{11})
Synergistic ratio[c]	6.9×10^2	0.8

[a] PIB: polyhedral inclusion body.
[b] Ninety-five percent confidence limit.
[c] LC of *Btk*/LC of (*Sl*NPV-*Btk*).

dropped as well. There was a substantial overlap in the 95% confidence limits of the two treatments at both LC_{50} and LC_{95}.

The synergistic effect of *Btk* by *Sl*NPV was investigated too (Table 2). It is clear that the addition of *Sl*NPV generally increased the toxic effect of *Btk* at a low concentration, where the LC_{50} outstandingly decreased from 2.5×10^5 spores/ml (for *Btk*) to 3.6×10^2 mixed polyhedra and spores/ml (for *Sl*NPV-*Btk* combination), indicating a synergistic ratio of 6.9×10^2. There was no substantial overlap in the 95% confidence limits of the two treatments. However, at high concentration, no clear synergism (an antagonism) was observed at LC_{95} since it increased from 1.3×10^7 spores/ml (for *Btk*) to 1.6×10^7 mixed polyhedra and spores/ml (for *Sl*NPV-*Btk* combination), indicating a synergistic ratio of 0.8. There was a substantial overlap in the 95% confidence limits of the three treatments (Tables 1 and 2).

Discussion and conclusions

The present study proved that laboratory isolate of *Sl*NPV could be transmitted to and infect 3rd larval instars of *Cx. pipiens*. Mosquito larval instars showed susceptibility to virus infection 24 h post-infection; where LC_{50} accounted for 1.3×10^3 PIB/ml. Generally, NPVs are considered of narrow host range to one species of insect or one genus at the most, but all within one type of the five insect orders (Lepidoptera, Hymenoptera, Diptera, Neuroptera and Trichoptera) (Martignoni, 1984). However, Bensimon et al. (1987) could prove that *Sl*NPV could be cross-transmitted to and from two species of locusts, provoked a lethal disease namely (Dark cheeks). Furthermore, an old study (Fukushi, 1935) postulated that the very same virus can propagate both in a plant host and in its leafhopper insect vectors. Bensimon et al. (1987) suggested that the evolutionary distance between the rice plant and a leafhopper is obviously much greater than the evolutionary distance between both insects.

Indeed, Van Beek and Hughes (1998) reported that there is relatively a long time between the NPV infection and the death of the infected insect, which was considered as a general disadvantage of the Baculovirus. In our study, however, the *Cx. pipiens* larval mortality was observed only 24 h post-infection with *Sl*NPV.

From results obtained, it appears that *Cx. pipiens* larvae were less susceptible to *Btk* treatment; when LC_{50} was 2.5×10^5 spores/ml. As mentioned before, the crystalline protein endotoxin of *Btk* is toxic to the larvae of many lepidopteran species. Yamamoto and McLaughlin (1981) reported that certain strain of *Btk* has also a "mosquito factor" toxic for both mosquito and Lepidopteran larvae, which is in consistence with our data. Moreover, Obha and Lee (2003) confirmed that certain *Btk* species from deer feces in Japan exhibited dual toxicity against *Bombyx mori* and *Aedes aegypti*. A study of Hassanain et al. (1997) recorded that *Btk* is potent even against certain species of soft and hard ticks.

B. thuringiensis is considered one of the most promising biocontrol agents. Its main drawback is that the mosquito larvicidal crystals of the bacteria do not persist for long periods in the environment due to their rapid inactivation by sunlight or other degradation agents (Schnepf et al., 1998). From laboratory evidence presented in this study and based on LC_{50}, it may be concluded the *Sl*NPV can be combined with *Btk* to achieve additive improved biological effect against *Cx. pipiens* mosquito larvae. Data showed a synergism between *Sl*NPV and *Btk*, where LC_{50} and LC_{90} of *Sl*NPV were decreased and synergistic ratios recorded were 3.6 and 1.3×10^2, respectively. Many authors have also reported synergistic interaction between NPV and *Bt*, targeting other insect pests, when applied simultaneously (McVay et al., 1977; Salama et al.,

1987, 1993; Padua et al., 1997; Shternshis et al., 2002; Knaak and Fiuza, 2005; Hesketh and Hails, 2008). It is noted that, in the present study, the LC_{50} of *Btk* was decreased upon combination with the virus (synergistic ratio = 6.9×10). However, there was an antagonism (0.8) recorded at LC_{95}. This result agreed with Salama et al. (1987) who report that the simultaneous application of *Sl*NPV and *Bt* showed an additive effect on *S. littoralis* larvae especially at lower concentrations.

In contrast to our results, Pingel and Lewis (1999) showed that mixtures of both entomopathogens (NPV, *Bt*) had no effect on insect mortality when compared with both pathogens separated or combined. Liu et al. (2006) observed that interaction between *Bt* and NPV varied according to the bioassay method and the concentration of the suspension. However, they concluded that most pathogen combinations showed an antagonistic effect. In the study of Matter and Zohdy (1981), an antagonistic effect was recorded while testing the biotic efficacy of NPV-*Bt* combination on the American bollworm larvae. However, they also observed that as larvae increase in age, the two pathogens interact synergistically.

The interaction between a pathogen and other compounds may be antagonistic due to decreased feeding or a change of gut pH (Pingel and Lewis, 1999) or each entity may act independently, leading to an additive mortality (McVay et al., 1977). In the present study, the *Sl*NPV-*Btk* combination was more effective against mosquito larvae than each pathogen alone. One hypothesis is that: in the presence of *Btk*, the number of insects that are able to escape NVP infection is reduced (through a combination of changes in host feeding behavior and delay in onset of host developmental resistance) (Hesketh and Hails, 2008). An alternative explanation is the possibility that the initial destruction by the *Bt* toxin facilitated the penetration and entry of the virus (Salama et al., 1993). Histopathological study of Knaak and Fiuza (2005) showed that after 6 h post-NPV-*Btk* treatment of certain lepidopteran caterpillars, the action of NPV was intensified when used in association with *Btk* causing intense vacuolization of the cytoplasm of larval midgut, causing cellular disorganization. Moreover, physiological study of Duraimurugan et al. (2009) confirmed that combined treatment of NPV and *Bt* results in suppression of detoxification enzymes in cotton bollworm larvae. This hypothesis is needed to be investigated for mosquito larvae as well.

Finally, the present study aimed at harvesting the benefits of *Sl*NPV-*Btk* combination, which was more effective against *Cx. pipiens* larvae than either *Sl*NPV or Btk alone. Larval mortality took place only 24 h post-infection. Using *Sl*NPV-*Btk* mixture at a low concentration could be a promising option for *Cx. pipiens* larvae control management.

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